

Lineage-Specific Identification of Nonhematopoietic Neoplasms by Flow Cytometry

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Abstract

To extend flow cytometry (FC) to the diagnosis of nonhematopoietic neoplasms, we have developed new flow cytometric assays to identify expression of cytokeratin, epithelial cell adhesion molecule (EpCAM)/epithelial glycoprotein-2, myogenin, and CD99. To validate these assays, we correlated the flow cytometric results with the histologic and immunohistochemical results on paraffin-embedded tissue in a series of 21 cases, including 17 carcinomas, 1 atypical carcinoid, 2 rhabdomyosarcomas, and 1 Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET). Six of 7 assayed carcinomas and the carcinoid were positive for cytoplasmic cytokeratin by the flow cytometric assay. EpCAM was expressed by 11 of 12 carcinomas that were assayed by FC. Both rhabdomyosarcomas expressed myogenin by FC, and the ES/PNET case expressed CD99. Interestingly, the blast-associated antigen CD90 was expressed uniformly on the ES/PNET case and on subsets of cells in the rhabdomyosarcoma and carcinoma cases. Potential applications of the flow cytometric assay to nonhematopoietic neoplasms will include evaluating samples with limited material, monitoring disease persistence and recurrence in patients with previous diagnoses, and making rapid diagnoses in urgent cases.

Recent technological advances in flow cytometry (FC) have enabled the rapid immunophenotyping of hematopoietic neoplasms, thereby revolutionizing the field of hematopathology. Although FC has become routine in diagnosing hematopoietic malignant neoplasms, it currently has virtually no role in the identification of epithelial or mesenchymal neoplasms. A review of the literature reveals rare use of flow cytometric immunophenotyping to aid in identifying nonhematopoietic neoplasms (NHNs).¹⁻⁵

Kussick et al² previously reported a series of nonhematopoietic small round blue cell tumors that were CD45– and CD56+ by FC. Since that study, we have developed new flow cytometric assays to assign lineage to NHNs, using fluorescent dye–conjugated antibodies to the following proteins: cytokeratin, epithelial cell adhesion molecule (EpCAM), myogenin, and CD99.

Cytokeratin and, more recently, EpCAM have been used to establish epithelial cell lineage. Cytokeratins are a group of intermediate filament proteins in the cytoskeleton of epithelial cells, which are absent in mesenchymal and hematopoietic cells. EpCAM, also known as epithelial surface antigen and epithelial glycoprotein 2 (EGP-2), is a 40-kd glycoprotein expressed in nonsquamous epithelia and associated neoplasms.⁶ Monoclonal antibodies recognizing EpCAM include AUA1, MOC31, GA733, KS1/4, and others.⁷ EpCAM is strictly expressed on the cell surface of human adult epithelial cells and is expressed in many epithelial neoplasms, including lung, prostate, breast, cervical, and colorectal carcinomas.⁷ It is important to note that mesothelioma is reportedly negative for EpCAM,⁸⁻¹⁰ which may make this a useful antigen for distinguishing mesothelioma from carcinoma.

Myogenin, a nuclear transcriptional regulatory protein, is expressed in early fetal skeletal muscle differentiation but it is absent in normal skeletal muscle. Immunohistochemical studies have shown that myogenin is a highly sensitive and specific marker for the diagnosis of all subtypes of rhabdomyosarcomas.^{11,12} The detection of intracellular myogenin by FC has not been previously described.

CD99 is a cell surface glycoprotein that has been found on numerous neoplasms, including Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET), lymphoblastic lymphomas/acute leukemias of both B- and T-cell types, desmoplastic small round cell tumor, mesenchymal chondrosarcoma, rhabdomyosarcoma, and others.¹³ Although the identification of strong expression of CD99 is not lineage-specific, its presence on a nonlymphoblastic, CD45- cell population is abnormal and suggestive of an NHN. The use of FC to document CD99 expression by ES/PNET has not been described previously.

An additional antigen found useful in diagnosing NHNs is CD56, also known as neural cell adhesion molecule (NCAM). CD56 is a cell surface protein that participates in homophilic interactions with CD56 molecules on other cells. CD56 is expressed by a variety of normal cell types, including a range of neuroectodermal derivatives, and natural killer cells. In addition, CD56 is expressed in a number of epithelial neoplasms, including small cell carcinomas,¹⁴ and in a variety of other neuroendocrine neoplasms.¹⁵ CD56 expression on NHNs has been reported by flow cytometric immunophenotyping of small cell carcinoma, ES/PNET, and neuroblastoma.^{1,2,16-18}

In the present article, we first describe a series of 35 NHNs that were analyzed by FC without lineage-specific immunophenotyping, using antibodies to CD45, CD56, and other antigens; results previously have been reported for a subset of these cases.² We then demonstrate the feasibility of using FC to assign lineage to 21 NHNs from a variety of anatomic sites, including carcinoma, rhabdomyosarcoma, and ES/PNET. To our knowledge, this is the first report in which FC has been used to demonstrate myogenin expression in rhabdomyosarcoma and CD99 expression in ES/PNET.

Materials and Methods

Case Identification and Assay Development

This study was motivated by our observation that nonhematopoietic tumor cells could be identified by FC as CD45- populations in tissues initially submitted to rule out lymphoma. In many of these cases, particularly those with small blue round cell morphologic features, the tumor cells

expressed bright CD56. In the first part of this study, we identified the spectrum of NHNs in which we could detect tumor cells as CD45-/CD56+ populations by FC. We retrospectively reviewed the database of the University of Washington Hematopathology Laboratory (Seattle) for the 1998-2002 period and identified 35 such cases in which subsequent histologic and/or immunohistochemical evaluation was diagnostic of an NHN, but in which lineage-specific antigens were not evaluated by FC.

The results from these 35 cases demonstrated that we could detect significant nonhematopoietic cell populations by FC. We therefore proceeded to develop flow cytometric assays to the epithelial markers cytokeratin and EpCAM/EGP-2, to the skeletal muscle marker myogenin, and to the cell surface marker CD99. The goal was to use these reagents for the definitive diagnosis of carcinoma, rhabdomyosarcoma, and ES/PNET, respectively.

In developing the assay to cytokeratin, we were able to use benign serous fluids from the University of Washington hematology and hematopathology laboratories as sources of positive (mesothelial) and negative (leukocyte) control cell populations for testing the cytokeratin antibody, in addition to an isotype-matched antibody control. The EpCAM assay was developed largely on epithelial malignant neoplasms, with leukocytes serving as internal negative controls, in addition to the isotype-matched antibody control. The myogenin assay was developed using the rhabdomyosarcoma cell line RD (kindly provided by Stephen Tapscott, MD, PhD, Fred Hutchinson Cancer Research Center, Seattle, WA)¹⁹ as a positive control and the hematopoietic cell lines K562 (a chronic myelogenous leukemia cell line) and RL7 (a follicular lymphoma cell line) as negative controls. The CD99 assay was developed using the 3 t(11;22)+ ES/PNET cell lines SK-ES, SK-N-MC, and RD-ES (kindly provided by Liu Yang, PhD, VA Medical Center, Seattle, WA)²⁰ and a bone marrow specimen involved by precursor T-lymphoblastic leukemia/lymphoma, as positive controls. The acute myeloid leukemia cell line YNH-1 (also a gift from Dr Yang)²¹ was used as a negative control in developing the CD99 assay.

In the second part of the study we prospectively identified 21 CD45- NHNs from June 2001 to October 2002 whose lineage we could positively identify by FC.

Flow Cytometric Analysis

To enable the second part of the study, ie, the assignment of lineage to NHNs, we developed flow cytometric assays to identify the following: (1) intracellular cytokeratin using the fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody J1B3 (Beckman Coulter [BC], Hialeah, FL); (2) cell surface epithelial cell adhesion molecule/EGP-2 using the FITC-conjugated monoclonal antibody EBA-1

(Becton Dickinson [BD], San Jose, CA); (3) intracellular myogenin using monoclonal antibody F5D (BD), which we conjugated to the FITC-like molecule Alexa-Fluor 488 (Molecular Probes, Eugene, OR); and (4) cell surface CD99 using FITC-conjugated monoclonal antibody TU12 (BD).

A number of additional clones, many of which are part of our standard lymphoma immunophenotyping panel, were used in at least some of the assays (clones are given in parentheses when applicable: CD45-phycoerythrin (PE)-Texas red (CD45-ECD, J.33, BC), IgG1 and IgG2a-FITC isotype controls (BD), IgG1-PE isotype control (BD), IgG2a-PE-Cy5 isotype control (BC), CD2-FITC (T11, BC), CD3-ECD (HIT3a, BC), CD4-PE-Cy5 (13B8.2, BC), CD5-FITC (Leu1, BD), CD5-PE-Cy5 (BL1a, BC), CD7-PE (3A1, BC), CD8-PE-Cy5 (SFC121Thy2D3, BC), CD10-PE (HI10a, BD), CD19-ECD (HD237, BC), CD20-FITC (H299, BC), CD34-PE-Cy5 (581, BC), CD38-FITC (T16, BC), CD56-PE (NCAM16.2, BD), CD90-PE (5E10, BD), and CD117-PE (104D2, DAKO, Carpinteria, CA). In each case the amount of antibody used was based on either the manufacturer's suggestion or titration experiments to optimize the signal to noise ratio.

In all cases, 4-color FC was performed in our laboratory on Epics XL instruments (BC). For peripheral blood and bone marrow specimens, an ammonium chloride-based technique (150-mmol/L concentration of ammonium chloride) was used to lyse the great majority of the erythroid cells from the specimen before the addition of antibodies. To increase the number of cells for analysis from solid tumor specimens, these specimens were finely minced and strained through 40- μ m cell strainers (BD) during processing.

For assays involving cell surface antigens only, the cells were washed once in a solution of phosphate-buffered saline (PBS)/0.3% bovine serum albumin (BSA)/0.1% sodium azide, incubated with the relevant antibodies for 15 minutes at room temperature in the dark, washed again in PBS/BSA/azide, and fixed in paraformaldehyde before flow cytometric analysis. For assays involving both cell surface and cytoplasmic antigens, which included all assays for cytokeratin and myogenin expression, cells first were incubated with antibodies to the cell surface antigens for 15 minutes at room temperature in the dark. The cells then were washed twice in PBS/BSA/azide, fixed for 15 minutes at room temperature in the dark using reagent A of the Fix and Perm kit (Caltag, Burlingame, CA), washed twice in PBS/BSA/azide, incubated with the relevant cytoplasmic antibody in the presence of reagent B of the Fix and Perm kit for 30 minutes at room temperature in the dark, washed twice in PBS/BSA/azide, and fixed in paraformaldehyde before flow cytometric analysis. Since the anticytokeratin and antimyogenin antibodies were both of IgG1 isotype, an FITC-conjugated, negative IgG1 isotype control antibody

was used in parallel to confirm the validity of the cytokeratin or myogenin immunoreactivity.

The number of cellular events collected from each tube of cells and antibodies reflected the considerable variation in the cellularity of the specimen. When possible, 100,000 viable cells were analyzed per tube. However, in many specimens, particularly those derived from fine-needle aspirations or cerebrospinal fluid, the number of cells was substantially fewer.

The flow cytometric data were compensated and analyzed in a standardized manner on Macintosh G3 or G4 computers (Apple Computer, Cupertino, CA) using software developed in our laboratory (by B.L.W.). Events with very low forward scatter, which were likely to include degenerated cells, were excluded from the analysis by forward-side scatter gating. In most cases, the nonhematopoietic cell population of interest was distinguished from hematopoietic cells in the specimen by CD45-side scatter gating.

Histologic and Immunohistochemical Evaluation

Since the specimens originated from both the University of Washington system and outside institutions that refer specimens to the University of Washington Hematopathology Laboratory for FC, the histologic and immunohistochemical evaluations of these cases were divided between the University of Washington Medical Center and the outside institutions. For all of the outside cases, the outside pathology reports were obtained, and for some of these cases (but not all), we reviewed the outside histologic and immunohistochemical findings. For cases in which the outside histologic and immunohistochemical evaluations seemed adequate, no additional studies were performed. For a subset of the outside cases and for all cases from the University of Washington system, histologic and/or immunohistochemical examination was performed in the University of Washington histology and immunohistochemistry laboratories, respectively.

For histologic evaluation, formalin-fixed, paraffin-embedded tissues generally were used. We evaluated 4- μ m sections by H&E staining. For immunohistochemical analysis, 4- μ m sections from at least 1 representative paraffin block of each case were deparaffinized, rehydrated in graded alcohol, and subjected to heat-induced epitope retrieval. After cooling, the relevant monoclonal antibody was applied and incubated for 40 minutes. For immunohistochemical analysis, antibodies to the following antigens were used (clones are given in parentheses as applicable): pancytokeratin (AE1/AE3, Chemicon, Temecula, CA), low-molecular-weight cytokeratin (CAM 5.2, BD), cytokeratin 7 (OV-TL, DAKO), CD56 (123C3.5, Neomarkers, Fremont, CA), CD99 (013, Signet, Dedham, MA), CD117 (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), chromogranin

(LK2H10, Cell Marque, Hot Springs, AR), desmin (D33, Neomarkers), myogenin (F5D, BD), and synaptophysin (SV38, DAKO). Immunolocalization was performed via a modified avidin-biotin immunoperoxidase technique with nickel chloride–enhanced 3,3'-diaminobenzidine chromogen or via a streptavidin-biotin immunoalkaline phosphatase technique. Finally, sections were counterstained with methyl green or hematoxylin.

Results

The 35 CD45–/CD56+ NHNs identified in our retrospective evaluation of the University of Washington Medical Center Hematopathology Laboratory database included the following: poorly differentiated carcinoma with neuroendocrine features/small cell carcinoma, 23; seminoma, 2; primary brain neoplasm (glioma), 2; rhabdomyosarcoma, 2; melanoma, 2; desmoplastic small round cell tumor, 1; medulloblastoma, 1; medullary carcinoma of the thyroid, 1; and

renal cell carcinoma, 1. The features of these cases are described in **Table 1**. Interestingly, the 2 seminomas expressed low-level CD10 and variable low CD38, in addition to CD56. While CD10 expression has been described in some germ cell tumors,²² we have not studied enough seminomas by FC to know whether this is a reproducible immunophenotype for this neoplasm. Note that 24 of the 35 cases were received before the development of our first flow cytometric assay for cytoplasmic cytokeratin in June 2001. Among the 11 cases received after this date were 6 carcinomas. The reasons for not evaluating epithelial antigen expression in these 6 cases included one or more of the following: (1) insufficient material remaining after the diagnosis of non-Hodgkin lymphoma was ruled out; (2) excessive cellular degeneration that precluded evaluation for epithelial origin or rendered attempted evaluation noncontributory; or (3) a decision by the referring pathologist to limit the flow cytometric evaluation to simply ruling out non-Hodgkin lymphoma.

Our development of flow cytometric assays to cytokeratin, EpCAM, myogenin, and CD99 enabled definitive

Table 1
Cases Without Lineage-Specific Assignment by Flow Cytometric Analysis*

Case No./Sex/Age (y)	Anatomic Site	Surgical Pathology Diagnosis	Flow Cytometric Results
1/F/18	Neck mass	Rhabdomyosarcoma	CD56+
2/F/73	Liver	Small cell CA, C/W lung primary	CD56+
3/F/58	Neck mass	Small cell CA	CD56+
4/M/60	Scalene LN	Small cell CA	CD56+
5/M/35	Retroperitoneal mass	Desmoplastic small round cell tumor	CD56+
6/F/68	Mediastinal LN	Small cell CA	CD56+
7/F/39	Abdominal mass	Small cell CA	CD56+
8/F/39	Paratracheal mass	Neuroendocrine CA	CD56+
9/M/34	Mediastinal mass	Rhabdomyosarcoma	CD56+
10/F/56	Cervical LN	Small cell CA	CD56+
11/F/65	Axillary mass	Poorly differentiated neuroendocrine CA	CD56+
12/M/48	Neck mass	Medullary thyroid CA	CD56+
13/F/72	Supraclavicular mass	Small cell CA	CD56+
14/M/67	Subcarinal LN	C/W small cell CA	CD56+
15/F/77	Lung mass	Small cell CA	CD56+
16/M/82	Bladder/pelvic mass	Poorly differentiated neuroendocrine CA	CD56+
17/M/60	Parietal brain mass	Poorly differentiated CA	CD56+
18/F/51	Bone marrow aspirate	Small cell CA	CD56+
19/M/59	Epidural mass	Small cell CA	CD56+
20/F/64	Neck nodule (fine-needle aspirate)	C/W small cell CA	CD56+
21/F/28	Posterior fossa mass	Medulloblastoma	CD56+
22/F/58	Neck mass	Undifferentiated CA	Variable CD56+
23/M/53	Supraclavicular LN	Seminoma	CD10+/CD56+, low CD38
24/F/53	Mediastinal mass	Malignancy, favor small cell CA	CD56+
25/F/56	Mediastinal mass	C/W small cell CA	CD56+
26/M/38	Retroperitoneal mass	Seminoma	CD10+/CD56+, low CD38
27/F/79	Pleural fluid	Small cell CA	CD56+
28/F/60	Bone marrow aspirate	Small cell CA	CD56+
29/M/43	Renal mass	C/W renal cell CA	CD56+
30/M/59	Axillary LN	Malignancy, favor melanoma	Variable CD56+
31/F/69	Mediastinal mass	Small cell CA	CD56+
32/M/89	Pleural fluid	Small cell CA	CD56+
33/M/66	Frontal brain mass	Malignancy, favor glioma	CD56+
34/F/38	Temporal brain mass	Primitive neuroectodermal tumor	CD56+
35/F/33	Pleura	Melanoma	CD56+

CA, carcinoma; C/W, consistent with; LN, lymph node; +, positive.
* All CD45–.

Table 2
Cases With Lineage-Specific Assignment by Flow Cytometric Analysis*

Case No./Sex/Age (y)	Anatomic Site	Surgical Pathology Diagnosis	Flow Cytometric Results
1/F/65	Upper lobe lung mass	Atypical carcinoid	CD56+; CK+
2/F/45	Supraclavicular mass	Large cell neuroendocrine CA	CD56+; minimal CK+
3/M/95	Axillary LN (FNA)	Specimen lost at referring institution	CD56+; CK+
4/F/56	Cervical LN	Non-small cell CA	CD56-; CK+
5/M/52	Site not designated	Non-small cell CA	CD56-; CK+
6/F/49	Thigh mass	Ewing sarcoma/primitive neuroectodermal tumor	CD56+/CD90+/CD117+; CD99+
7/F/5	Upper arm mass	Rhabdomyosarcoma	CD56+; myogenin+
8/M/54	Parapharyngeal space	Small cell CA	CD56+; CK+
9/F/81	Submandibular LN	Small cell CA	CD56+; EpCAM+; CK+
10/M/64	Retroperitoneal mass	Small cell CA	CD56+; EpCAM+; CK-
11/F/3	Facial mass	Rhabdomyosarcoma	CD56+; myogenin+
12/F/75	Peripheral blood	Small cell CA	CD56+; EpCAM+
13/M/54	Pleural fluid	Small cell CA	CD56+; EpCAM+
14/F/57	Cervical LN	Non-small cell CA	CD56-; EpCAM+
15/M/50	Peribronchial mass (FNA)	Small cell CA	CD56+; EpCAM+
16/F/82	Pelvic mass (FNA)	Non-small cell CA	CD56-; EpCAM+
17/F/19	Cervical LN	Non-small cell CA	CD56-; EpCAM+
18/M/38	Paratracheal LN	Small cell CA	CD56+; EpCAM+
19/M/63	Thoracic mass	Small cell CA	CD56+; EpCAM+
20/F/64	Cerebrospinal fluid	Small cell CA	CD56+/CD90+; EpCAM-
21/M/46	Cerebrospinal fluid	Non-small cell CA	CD56-; EpCAM+

CA, carcinoma; CK, cytokeratin; EpCAM, epithelial cell adhesion molecule; FNA, fine-needle aspiration (all others were larger biopsy specimens); LN, lymph node; +, positive; -, negative.

* All CD45-.

lineage assignment in 21 subsequent cases with prominent CD45- cell populations from a variety of anatomic sites (Table 2). In these 21 cases, the initial flow cytometric analysis suggested the following diagnoses: 12 carcinomas with neuroendocrine features (strongly CD56+), 6 carcinomas without convincing neuroendocrine features (essentially CD56-), 2 rhabdomyosarcomas, and 1 ES/PNET. Wright-Giemsa-stained cytocentrifuged preparations were available for 11 of the 12 CD56+ carcinomas, and the cytologic features alone suggested that 8 of these 11 cases were small cell carcinomas, while 3 seemed to be neuroendocrine carcinomas of larger cell type.

The results of subsequent morphologic and immunohistochemical studies of this series, which were available for 20 of 21 cases, confirmed the flow cytometric findings in all 20 cases. The final diagnoses included the following: small cell carcinoma, 9; large cell neuroendocrine carcinoma, 1; atypical carcinoid, 1; non-small cell carcinoma, 6; rhabdomyosarcoma, 2; and ES/PNET, 1. Therefore, using histologic and immunohistochemical evaluation as the “gold standard,” the combination of FC and cytology correctly classified suspected neuroendocrine carcinomas as small cell carcinoma vs other neuroendocrine carcinoma/carcinoid in 10 (91%) of 11 cases; in the 11th case, the cytologic features somewhat favored a large cell neuroendocrine carcinoma, but the outside immunohistochemical diagnosis was small cell neuroendocrine carcinoma. Flow cytometric, histologic, and immunohistochemical findings from a representative small cell carcinoma and the atypical carcinoid are shown in

Image 1 and Image 2, respectively. Note that in 1 case of morphologically straightforward small cell carcinoma involving the cerebrospinal fluid (case 20, Table 2), the tumor cells showed the expected uniform expression of CD56, in addition to strong expression of the stem cell-associated antigen CD90, but were negative for EpCAM (cytoplasmic cytokeratin was not evaluated in these cells owing to the small sample). Thus, EpCAM does not seem to be expressed uniformly in small cell carcinomas.

Just as CD56 positivity predicted neuroendocrine morphologic findings among the epithelial neoplasms, the lack of CD56 expression predicted the diagnosis of non-small cell carcinoma. Image 3 illustrates a representative case of metastatic poorly differentiated adenocarcinoma from a cervical lymph node.

Our flow cytometric diagnoses of carcinoma were not limited to samples from soft tissue masses and serous fluids. In case 12 (Table 2), FC enabled a primary diagnosis of small cell carcinoma to be made on EpCAM+/CD56+ cells in the peripheral blood. This diagnosis was confirmed by histologic and immunohistochemical studies on tissue obtained at autopsy. In case 21 (Table 2), a primary diagnosis of non-small cell carcinoma was made on morphologically atypical EpCAM+ cells in the cerebrospinal fluid. The diagnosis of non-small cell carcinoma was confirmed subsequently by transbronchial biopsy of an endobronchial lung mass.

The flow cytometric results from 1 of the 2 cases of rhabdomyosarcoma are shown with the corresponding histologic

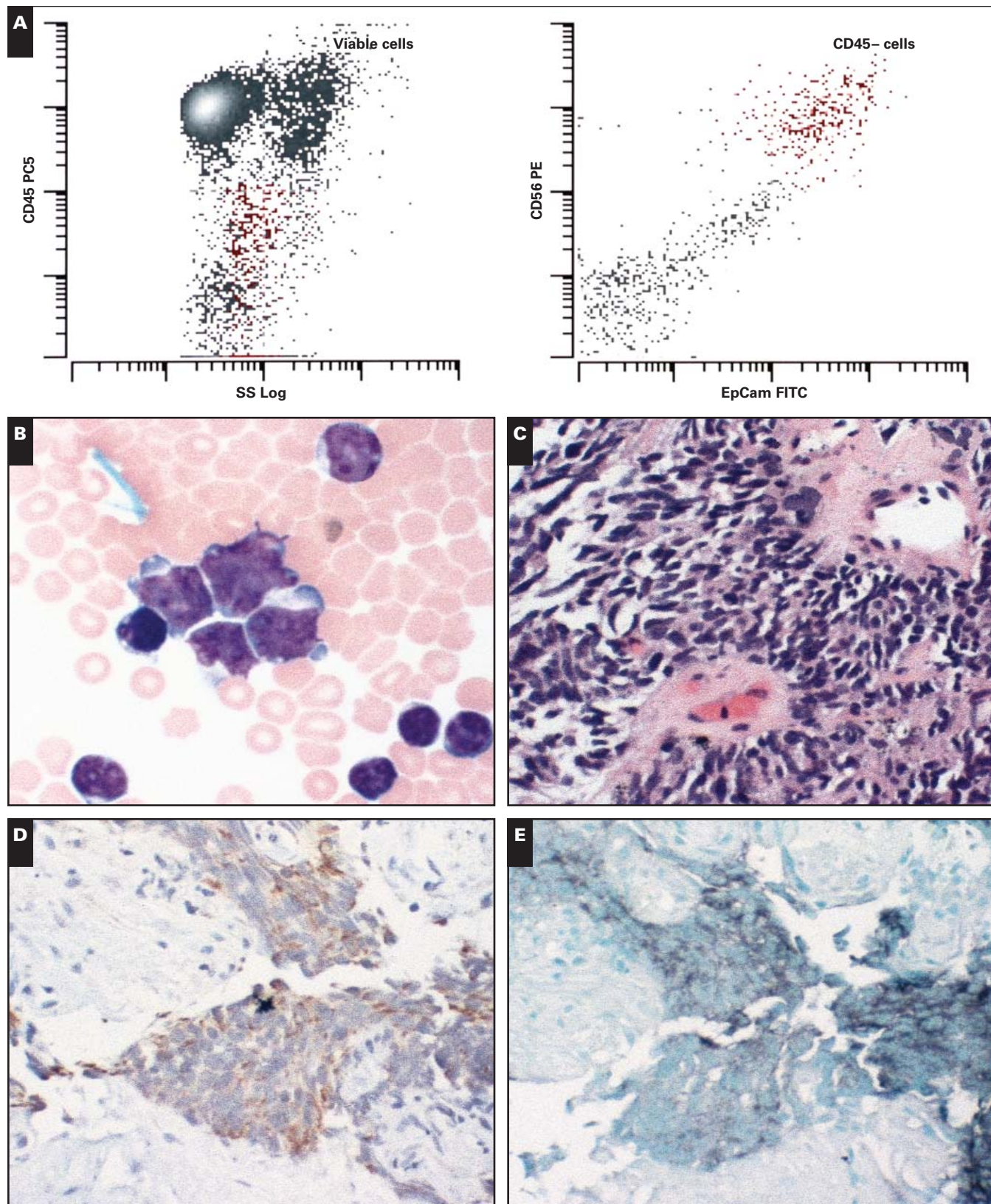


Image 1 Small cell carcinoma/neuroendocrine carcinoma. **A**, Flow cytometric analysis performed on pleural fluid showing the neoplastic cells in red, with a large number of brightly CD45+ lymphocytes and macrophages in the specimen. **B**, Cytocentrifuged preparation of pleural fluid showing rare clusters of neoplastic epithelial cells (Wright-Giemsa, $\times 425$). **C**, Section of a subsequently biopsied lung mass (H&E, $\times 170$). **D**, Low-molecular-weight cytokeratin immunohistochemical analysis (cellular adhesion molecule 5.2, $\times 85$). **E**, Synaptophysin immunohistochemical analysis (synaptophysin, $\times 85$). EpCAM, epithelial cell adhesion molecule; FITC, fluorescein isothiocyanate; PC5, PE-Cy5; PE, phycoerythrin; SS, side scatter.

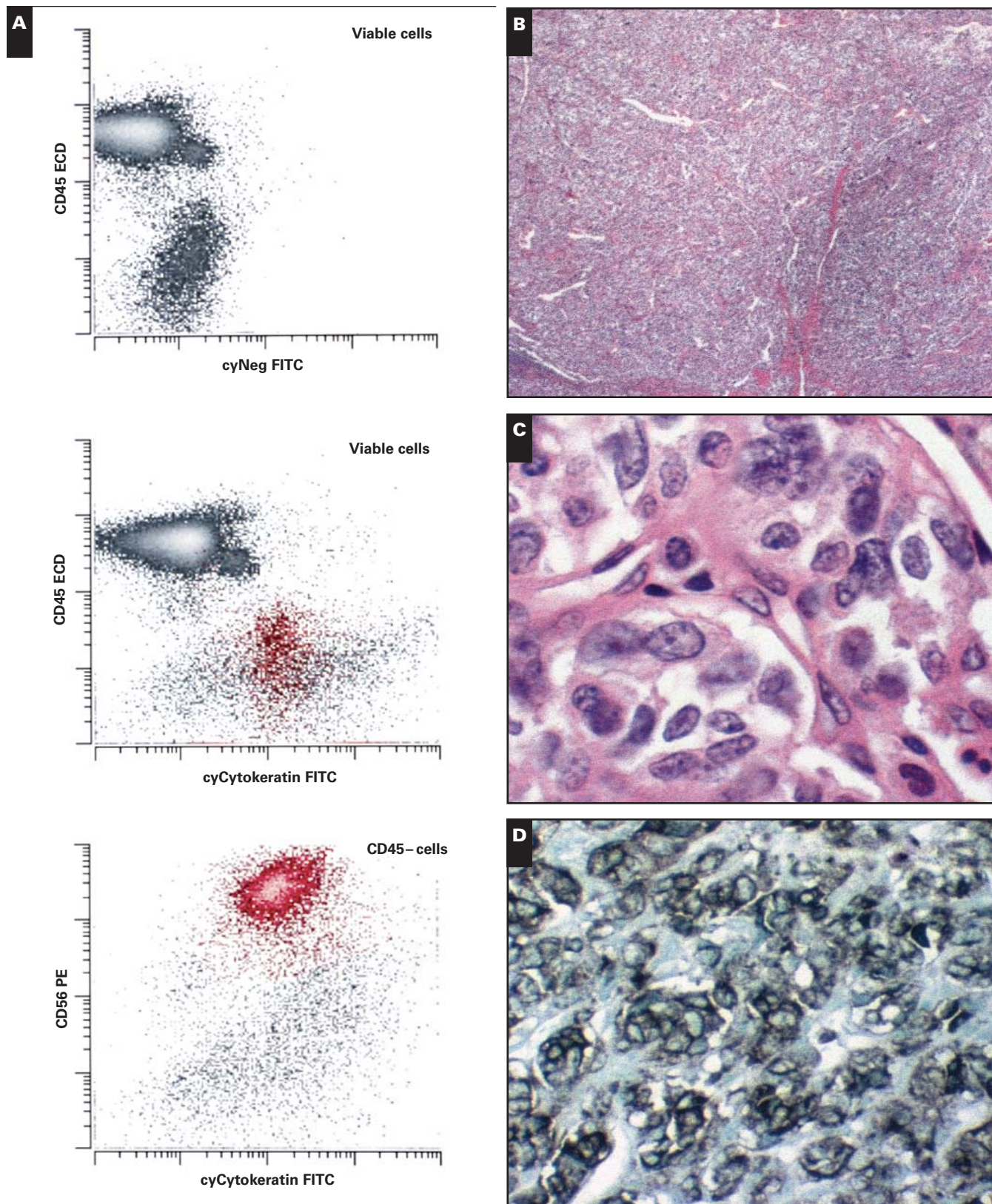


Image 2 Atypical carcinoid of the lung. **A**, Flow cytometric results showing the neoplastic cells in red. **B** and **C**, Stained sections of the atypical carcinoid (**B**, H&E, $\times 34$; **C**, H&E, $\times 425$). **D**, Chromogranin immunohistochemical analysis (chromogranin, $\times 170$). cyCytokeratin FITC, cytoplasmic cytochrome antibody; cyNeg FITC, negative cytoplasmic FITC antibody control; ECD, PE–Texas red; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

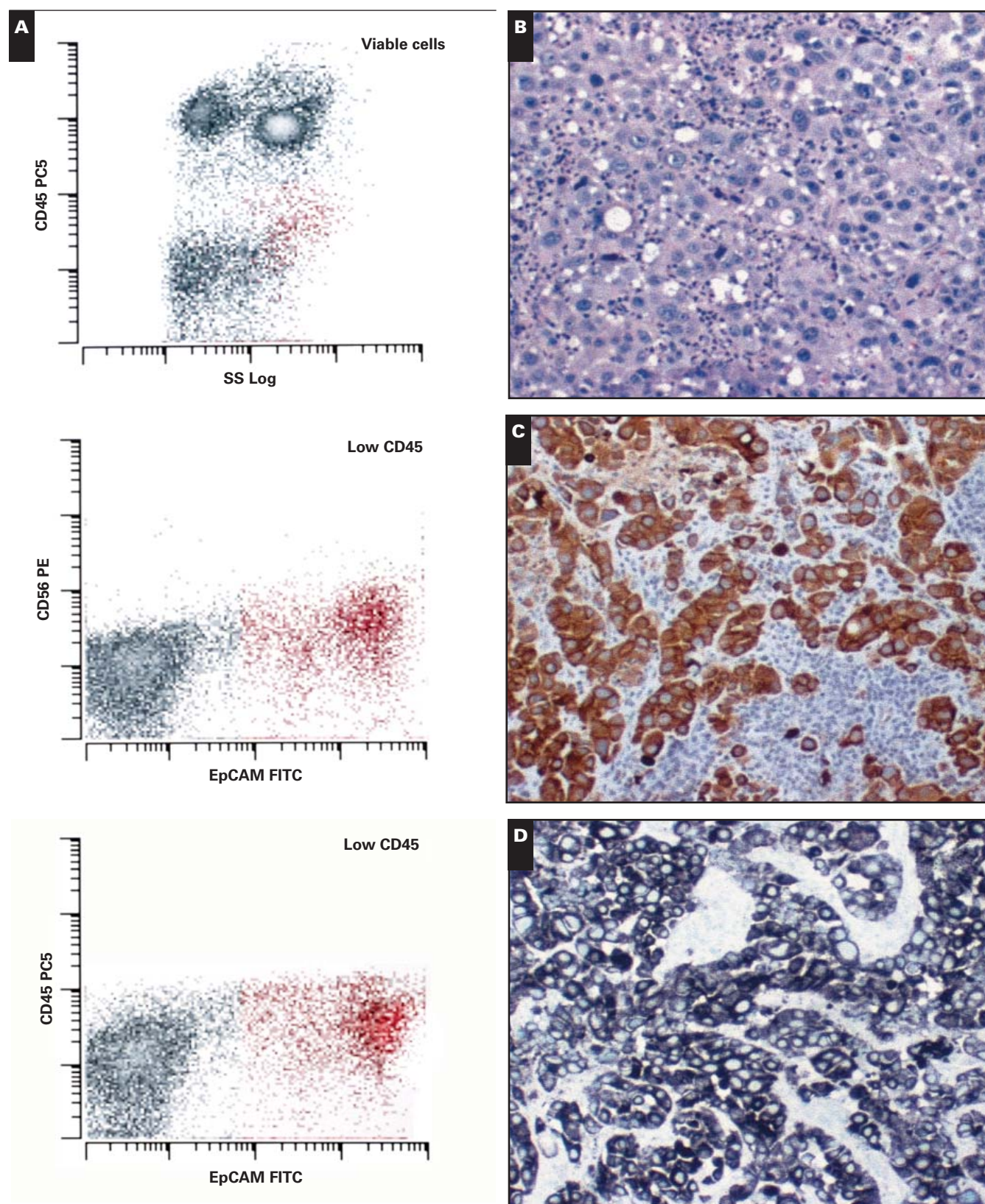


Image 3 Poorly differentiated non-small cell carcinoma. **A**, Flow cytometric results showing the neoplastic cells in red. **B**, Stained section of the non-small cell carcinoma (H&E, $\times 170$). **C**, Pancytokeratin immunohistochemical analysis (pancytokeratin, $\times 170$). **D**, Cytokeratin 7 immunohistochemical analysis (cytokeratin 7, $\times 170$). EpCAM, epithelial cell adhesion molecule; FITC, fluorescein isothiocyanate; PC5, PE-Cy5; PE, phycoerythrin; SS, side scatter.

and immunohistochemical findings in **Image 4**. In addition to expressing the skeletal muscle marker myogenin, the 2 cases of rhabdomyosarcoma in this series showed a spectrum of CD56 expression, with a variably prominent subset of the cells expressing bright CD56 in both cases. A similar pattern of CD56 expression also was identified in the 2 rhabdomyosarcoma cases from the initial series of 34 cases evaluated before we developed the flow cytometric assay for myogenin. Interestingly, subsets of cells in both myogenin+ cases showed expression of the stem cell–associated antigen CD90 in the flow cytometric assay (data not shown). Strong expression of CD56 (data not shown), as well as myogenin and CD90 (**Image 4B**), also was identified on the rhabdomyosarcoma cell line RD.

While a CD99+/CD45– immunophenotype is not entirely diagnostic of ES/PNET, in case 6 (**Table 2**) of this series the coexpression of low-intermediate CD99, low-intermediate CD56, intermediate CD117, and intermediate-bright CD90 was consistent with the diagnosis of ES/PNET. The results of the flow cytometric, histologic, and immunohistochemical studies for this case are shown in **Image 5**. The 3 ES/PNET cell lines SK-ES, SK-N-MC, and RD-ES showed a similar flow cytometric immunophenotype, but with extremely bright CD90 expression (data not shown). In addition to the well-known expression of CD99 in ES/PNET,¹³ expression of CD56¹⁷ and CD117²³⁻²⁶ has been identified in ES/PNET. However, to our knowledge, this represents the first report of CD90 expression in ES/PNET.

Discussion

While histologic and immunohistochemical evaluation is the acknowledged gold standard for the diagnosis of most NHNs in surgical pathology, FC has a variety of features that make it a useful adjunctive tool in the diagnosis of these neoplasms.

First, in comparison with histologic and immunohistochemical evaluation, FC is a rapid technique. When the initial panels of antibodies are negative for abnormal hematopoietic cell populations, we have found that minimal work is required to apply additional antibodies to rule out other entities, such as carcinoma or rhabdomyosarcoma. Even with additional flow cytometric studies to look for NHNs, the final results can almost always be obtained within 24 hours of receipt of the specimen, on the same day or early the following day. The rapidity of FC thus is ideal for clinically urgent cases, such as the diagnosis of mediastinal masses causing superior vena cava syndrome or masses causing spinal cord–related symptoms. Evaluating the same specimen by histologic and immunohistochemical techniques typically would take at least 48 hours from specimen

receipt, and frequently longer if additional immunohistochemical analysis was needed after the first round of antibodies was evaluated.

Second, because of the relatively great amount of immunophenotypic information on individual cells and because virtually all cells in a specimen can be evaluated, FC can facilitate diagnoses on paucicellular specimens that would be difficult or impossible by histologic and immunohistochemical methods.

Third, compared with immunohistochemical methods evaluated by the human eye, FC provides more objective measures of antigen expression. The dynamic range of fluorescence intensity and antigen expression that can be measured by modern flow cytometers is roughly 4 logs, which would seem to be a much broader range than that detectable by the human eye in the assessment of immunohistochemical results.

Fourth, because living cells with native antigen conformations are used in FC, antigen retrieval techniques generally are not required for flow cytometric evaluation, in contrast with immunohistochemical evaluation. Thus, antigens such as CD90 can be evaluated readily by FC, whereas we have been unable to detect this antigen reliably in formalin-fixed, paraffin-embedded tissue.

Finally, at our institution, the cost for a single antibody used in a flow cytometric assay is roughly one sixth of the per-antibody cost associated with immunohistochemical analysis.

The notion that FC may be unsuitable for “solid” tumors was not supported by our experience. Desmoplastic stroma or extensive fibrosis does not hinder the processing of these specimens. Simple mincing of the tissue sample was sufficient to prepare cell suspensions for adequate flow cytometric analysis. As the study progressed, when possible, we favored using the EpCAM antibody over the cytokeratin antibody, because the latter required a relatively more labor-intensive and time-consuming permeabilization procedure to permit the antibodies to bind to intracellular cytokeratin.

Although identifying a CD45– population expressing cytokeratin and/or EpCAM does not indicate, per se, whether the population is benign or malignant, putting the flow cytometric data in the appropriate morphologic and clinical context usually makes the final diagnosis obvious. For example, if a specimen is truly from a lymph node, then the identification of any prominent cytokeratin+ or EpCAM+ cell population would be abnormal and suggestive of metastatic carcinoma, even if the patient does not have a history of carcinoma. Similarly, in specimens such as serous fluids that normally contain CD56– epithelial (mesothelial) cells, the coexpression of CD56 with cytokeratin or EpCAM is highly suggestive of a malignant epithelial neoplasm. To broaden the usefulness of FC for identifying metastatic

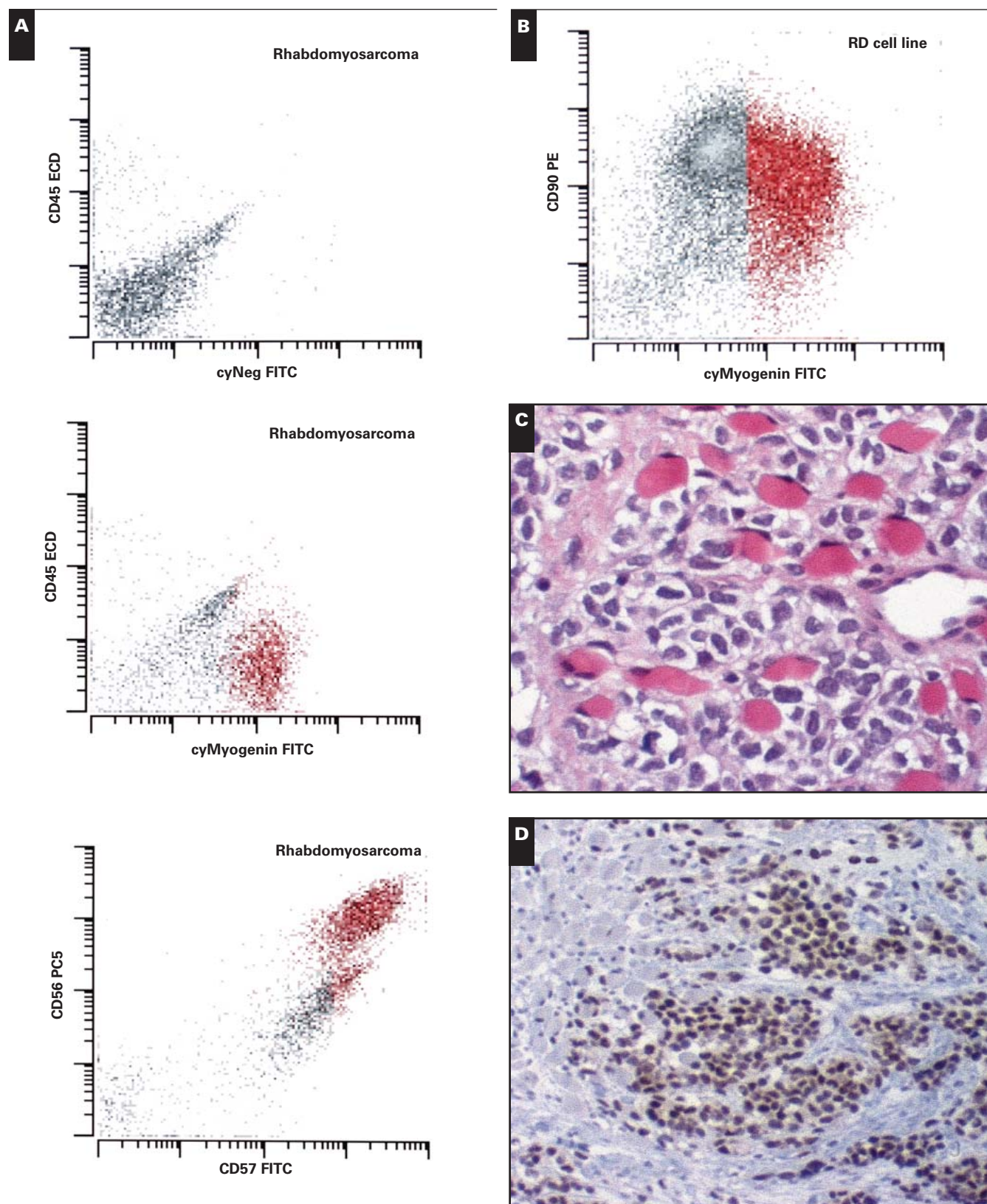


Image 4 Rhabdomyosarcoma. **A**, Flow cytometric results for a case from the series, showing the neoplastic cells in red. **B**, Flow cytometric analysis on the RD cell line showing acquisition of myogenin with a mild decline in CD90 expression (myogenin+ cells in red). **C**, Stained section of the rhabdomyosarcoma (H&E, $\times 170$). **D**, Myogenin immunohistochemical analysis (myogenin, $\times 85$). ECD, PE–Texas red; FITC, fluorescein isothiocyanate; PC5, PE–Cy5; PE, phycoerythrin.

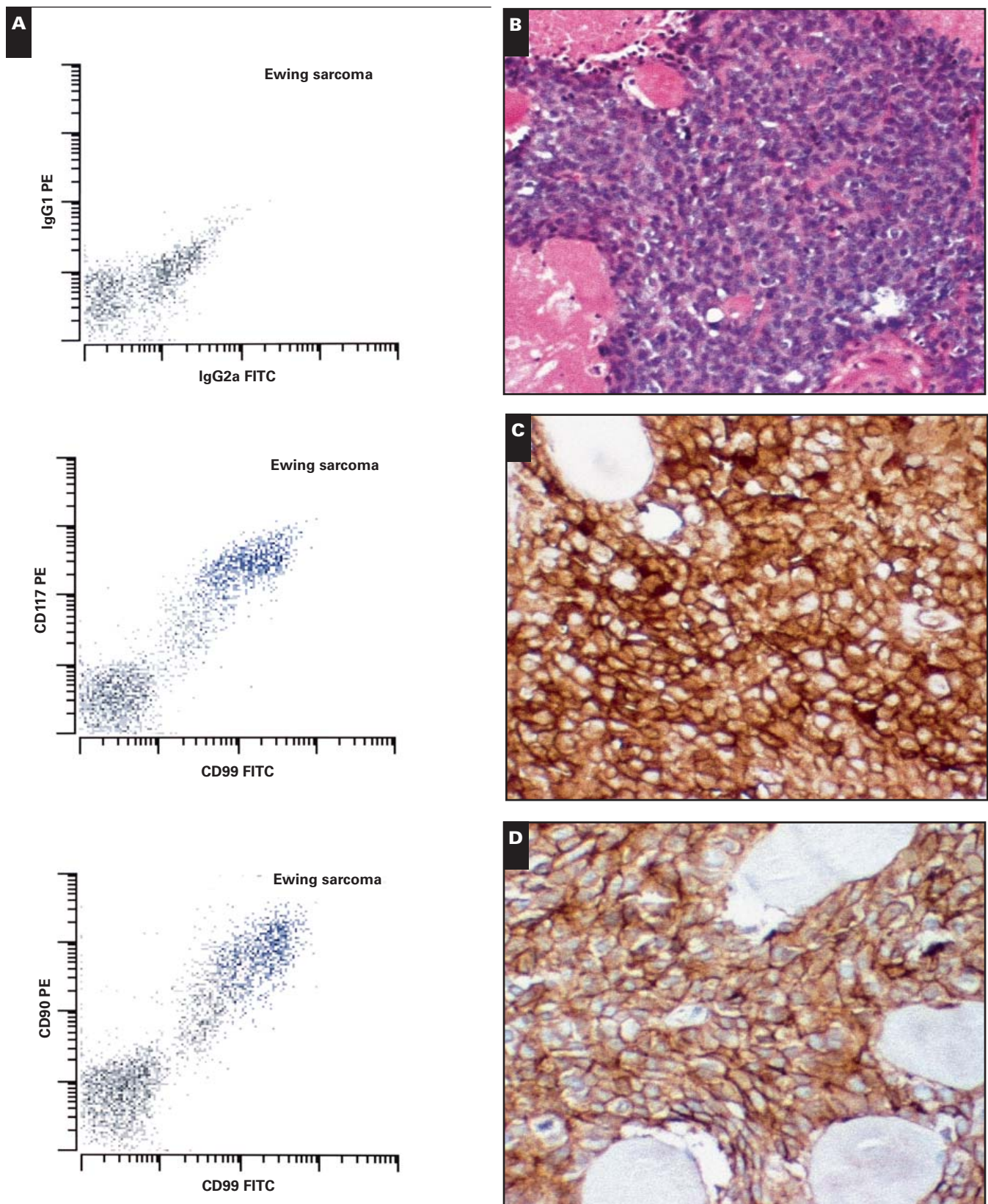


Image 5 Ewing sarcoma/primitive neuroectodermal tumor. **A**, Flow cytometric results showing the neoplastic cells in blue, with the dot plot in the top panel demonstrating the immunoreactivity with isotype-matched control mouse monoclonal antibodies. **B**, Stained section of the tumor (H&E, $\times 85$). **C**, CD99 immunohistochemical analysis (CD99, $\times 170$). **D**, CD117 immunohistochemical analysis (CD117, $\times 170$). FITC, fluorescein isothiocyanate; PE, phycoerythrin.

epithelial malignant neoplasms in serous fluids or other specimens in which there is likely to be a normal epithelial component, antibodies to tissue-associated proteins such as estrogen receptor, progesterone receptor, and prostate-specific antigen could be conjugated to fluorescent molecules. While the commercial availability of such conjugated antibodies currently seems limited, new reagents could be developed with the help of appropriate kits, such as those mentioned in the "Materials and Methods" section.

The main disadvantage of FC is the absence of contextual information about a tumor, such as its tissue architecture and histologic features and its relationship to surrounding structures, including the surgical margins. Thus, flow cytometric immunophenotyping of NHNs does not obviate the need for morphologic evaluation of a specimen at the time of initial diagnosis of a neoplasm. However, because the combination of flow cytometric and cytologic evaluation was able to assign lineage correctly in all 20 cases on which we had histologic data, the combination of FC and cytology would seem to be a reasonable first-line technique for confirming recurrence or metastasis of a previously diagnosed tumor.

Because we do not have follow-up information about all specimens with normal hematopoietic cell populations that we have evaluated by FC since 1998, we are not certain about the sensitivity of FC for detecting NHNs. The inclusion of CD45 and CD56 among the antigens we assess routinely in lymph node specimens makes it likely that we would detect (as CD56+/CD45- populations) virtually all cases in which substantial populations of viable neuroendocrine or small cell carcinomas are present. As indicated in Table 2, neither the anti-EpCAM antibody (negative on case 20) nor the anticytokeratin antibody (virtually negative on case 2) used here are likely to be 100% sensitive for neuroendocrine and small cell carcinomas. However, the cytologic features of these cases, in combination with the CD56+/CD45- immunophenotype, generally will be strongly suggestive of neuroendocrine and small cell carcinomas, particularly when the clinical history argues against some other CD56+ small blue round cell tumor. Because the CD56 positivity that led us to suspect neuroendocrine or small cell carcinoma after running our typical lymphoid flow cytometric panel generally will not be present in non-small cell carcinoma, our overall ability using FC to detect non-small cell carcinoma may be less than our ability to detect neuroendocrine and small cell carcinomas. Again, the overall sensitivity of the assay for non-small cell carcinoma is likely to be enhanced substantially by morphologic evaluation of cytocentrifuged preparations of the specimen, which likely would reveal atypical epithelioid cell populations if these are present in substantial numbers.

FC is widely accepted for the detection of minimal residual disease in hematopoietic neoplasms and, with the

proper antibody combinations, FC is well suited for detecting residual NHNs. For example, Komada et al¹ showed that FC was superior to conventional cytology for detecting 1 neuroblastoma cell in 10⁴ to 10⁵ other cells in peripheral blood or bone marrow samples from pediatric patients. Nagai et al¹⁸ showed similar findings using a modified combination of antibodies. The detection of minimal residual disease also has been demonstrated in peripheral blood samples from patients with renal cell carcinoma²⁷ or prostatic carcinoma.²⁸ Flow cytometric analysis of sentinel lymph nodes from patients with breast carcinoma revealed sensitive detection of micrometastasis that occasionally was superior to H&E and immunohistochemical staining.²⁹

Our identification of surprisingly bright CD90 on several neoplasms and the rhabdomyosarcoma and ES/PNET cell lines is intriguing, as CD90 is known to be expressed on subsets of hematopoietic³⁰ and nonhematopoietic stem cells.^{31,32} Therefore, the expression of CD90 in the ES/PNET case and cell lines, in the rhabdomyosarcoma cases and cell line, and in subpopulations of several of the carcinomas raises the possibility that strong CD90 expression identifies a highly proliferative and/or stem cell-like component of these neoplasms. The observation of uniform CD90 on the cell lines, in contrast with the lower levels of expression in the actual human tumors in this series, further suggests that the CD90+ population may be selected for when cell lines are made from such tumors. The data from the RD cell line (Image 4B), which demonstrate a decline in CD90 expression as cells acquire the differentiation-associated antigen myogenin, would be consistent with the hypothesis that the most strongly CD90+ cells may represent the "stem-cell" component of the overall population. However, since there are virtually no published immunophenotypic characterizations of the stem cells in NHNs and since some studies have suggested that the leukemic stem cells in acute myeloid leukemia actually are CD90-,³³ future immunophenotypic characterization of the putative stem cells from a variety of NHNs will be required to determine the significance of CD90 expression in these neoplasms.

Flow cytometric immunophenotyping is underused as a diagnostic tool for NHNs. With the development of advanced flow cytometers and more fluorescent dye-conjugated antibodies, this technique will become even more powerful as more antigens can be analyzed simultaneously on specific cell populations. If our hypothesis that CD90-expressing subpopulations are likely to represent the neoplastic stem cells in certain human tumors turns out to be correct, then purification and characterization of such subpopulations by FC/cell sorting may enable the development of novel therapies targeted to these important subpopulations. From a diagnostic viewpoint, future applications of FC to

NHNs should include residual disease detection, evaluation of samples with limited material (such as fine-needle aspirates and other cytologic specimens), and generation of rapid diagnoses in clinically urgent cases.

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